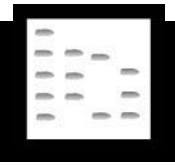
INCLUSION COMPLEXATION: LIQUID CHROMATOGRAPHY



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Introduction

Liquid chromatography (LC) may be simply described as an analytical technique used to separate the individual components of a solution based on their relative affinities for a liquid mobile phase and a stationary phase. There are numerous modes of LC (i.e. reversed-phase, normal-phase, ion exchange) that are commonly classified according to the mechanism by which separation occurs. One such mechanism involves the formation of inclusion complexes. An inclusion complex can be defined as an entity, consisting of two or more molecules, in which one molecule, the 'host', noncovalently encapsulates or includes a 'guest' molecule. In chromatography, differences in the strength of binding between guest molecules or analytes and a specific host allow for separation. The purpose of this article is to review recent applications of inclusion complexation in liquid chromatography. Although a variety of small molecule interactions with macromolecules or polymers have been ascribed to inclusion complexation, the focus of this article is on well-defined host molecules.

Host Molecules

Currently, there is a variety of compounds that can serve as a host molecule in an inclusion complex, most of which may be grouped into one of four classes. These classes include cyclodextrins, crown ethers, calix[*n*]arenes and macrocyclic antibiotics. A representative structure from each class of hosts is shown in **Figure 1**. One characteristic shared by each class of host molecules is the ability to include selectively compounds on the basis of size and shape.

It is important to point out that many inclusion host molecules possess at least one chiral centre (e.g. macrocyclic antibiotics and cyclodextrins) and that the vast majority of applications lie in the area of chiral separations. Chiral molecules are those that can exist as nonsuperimposable mirror images, or enantiomers. It has been demonstrated that the enantiomers of many chiral compounds exhibit different bioactivities and/or biotoxicities. It has also been demonstrated that enantiomers may exhibit different complex stabilities when included in a given chiral host. This fact, combined with an increased awareness of the implications of chirality, has led to the exploitation of inclusion complexation for chromatographic chiral separations. For this reason the emphasis of this article is placed on chiral applications, but some achiral applications are also presented.

Briefly, cyclodextrins (CDs), which are natural products, are cyclic oligosaccharides consisting of D-glucose rings linked through α-(1, 4)-bonds. The glucose units are arranged in a toroidal structure best described as a truncated cone. The most common cyclodextrins are α-, β- and γ-CD which consist of six, seven and eight glucose rings, respectively. The interior of the cyclodextrin molecule is hydrophobic while the exterior is hydrophillic. Because a cyclodextrin consists of chiral D-glucose units, it constitutes a chiral host.

Crown ethers are perhaps best described as synthetic, heterocyclic macrocycles with repeating units of $(-X-C_2H_4-)$ where X is the heteroatom. In contrast to cyclodextrins, crown ethers are characterized by a hydrophobic exterior and a hydrophilic cavity. The cavity typically has a strong affinity for cationic species owing to electrostatic interactions between the cation and the heteroatoms of the crown ether. These characteristics of crown ethers have led to their use as phase transfer catalysts in organic synthesis.

Calix[*n*]arenes are synthetic, cyclic oligomers composed of phenolic units linked by methylene bridges. They may contain four to eight aryl moieties forming a macrocyclic structure with a central cavity. The inclusion interaction between calixarenes and various guest molecules is determined both by the cavity size and by the nature of the functional groups that act as binding sites. Although still relatively unexplored, the ability to functionalize calixarenes offers yet another method for the tailoring of chromatographic selectivity.

Finally, macrocyclic antibiotics typically possess numerous chiral centres that surround several pockets or cavities bridged by a series of aromatic rings. Like cyclodextrins, the macrocyclic antibiotics are also natural products. In contrast to other host molecules, native macrocyclic antibiotics incorporate ionizable moieties.

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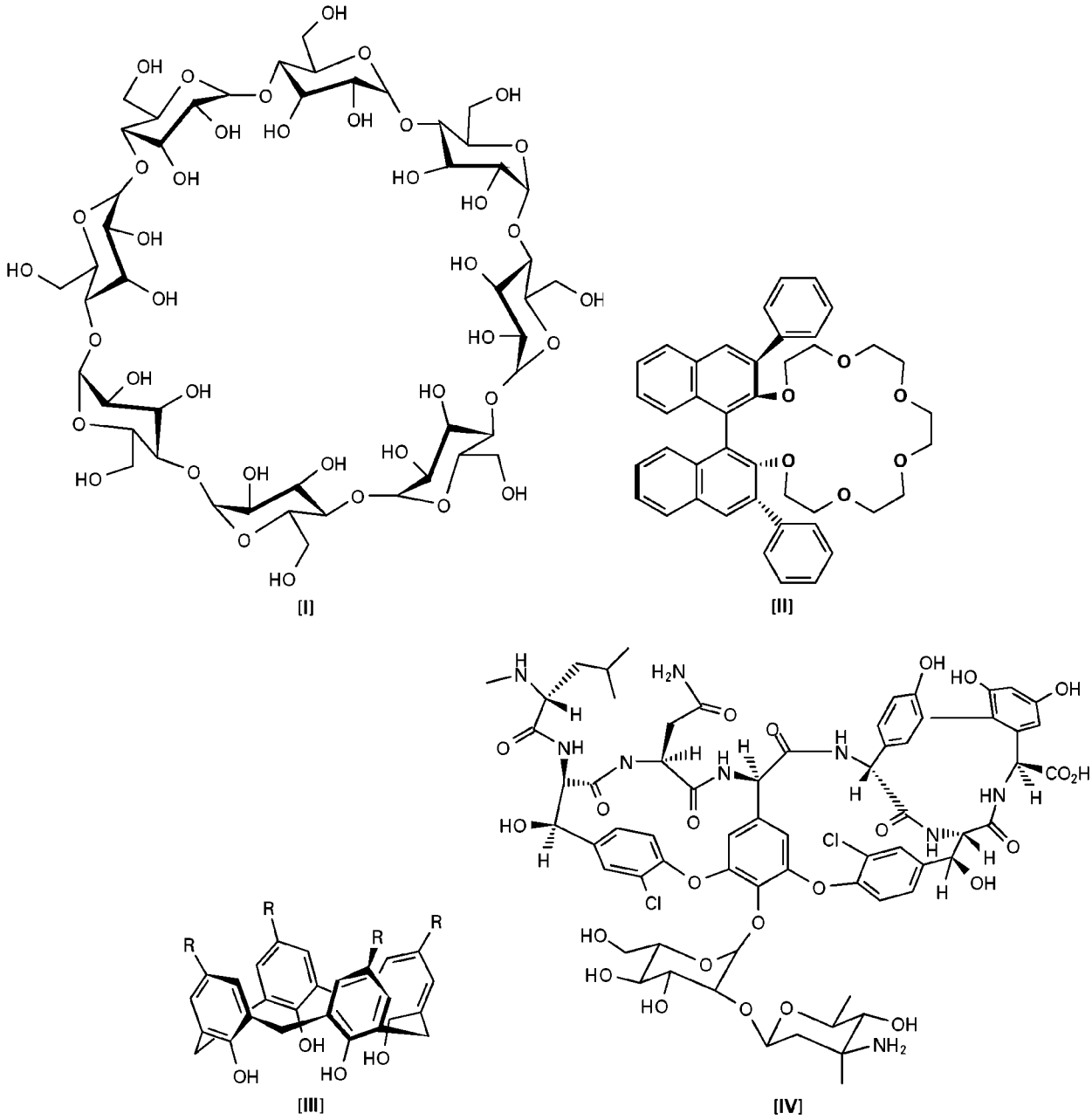

Figure 1 Representative structures of inclusion complex host molecules: [**I**], γ-CD; [**II**], a chiral crown ether; [**III**], a calix[4]arene; and [**IV**], vancomycin.

Currently there are two primary methods for exploiting inclusion complexation in LC. One method is the use of an inclusion host molecule as an immobilized ligand on a chromatographic sorbent. Stationary phases modified by cyclodextrin, crown ether, calixarene and macrocyclic antibiotic have all been reported. Alternatively, inclusion complex host molecules may be employed as mobile phase additives.

Historically, many of the hosts used to modify stationary phases originated as mobile phase additives, and thus will be considered first. Some advantages and disadvantages of each method are outlined in **Table 1**.

Inclusion Complexation in the Mobile Phase

One approach to exploiting inclusion complexation in chromatography involves dissolving the host in the mobile phase. As indicated in Table 1, use of the host as a mobile-phase additive (MPA) instead of the cor-

Table 1 Advantages and disadvantages of using inclusion complex host molecules as mobile phase additives

Advantages	*Disadvantages/limitations*
Less expensive, conventional packed columns can be used	Possible interference with detection of analyte
Type and concentration of host are easily changed	Potential solubility problems
Wider variety of additives compared with stationary phases	Effect on mobile phase viscosity
Different selectivities relative to corresponding stationary phase	Impact on analyte recovery
	Large amounts of additive needed for column pre-equilibration

responding bonded stationary phase has a number of advantages and disadvantages, depending on the application. One of the primary reasons for utilizing the host as a MPA is that it offers more flexibility for the application, both in the type of host used and the concentration of the host. Unfortunately, the amount of additive needed to equilibrate chromatographic columns and to separate the samples can be very large, making this approach very costly. This is not a problem in thin-layer chromatography, since TLC plates do not require equilibration with the mobile phase. In either case, the presence of the additive in the mobile phase can complicate detection and analyte recovery.

Both cyclodextrins and macrocyclic antibiotics have been used extensively as MPAs, with cyclodextrins being the most widely used. Calixarenes and crown ethers have found more limited use as MPAs. The large absorption of the phenyl groups in the calixarene molecule makes it very difficult to detect the analyte by UV spectrophotometry owing to the high background. Crown ethers have been used to aid in the dissolution of buffers in strong organic systems and to assist in the partitioning of analytes into the mobile phase. In one study, 18-crown-6 was used in the separation of carboxylated porphyrins. It was suggested that the crown ether could facilitate the partition of the porphyrins into the mobile phase through hydrophobic and polar interactions.

Cyclodextrins

Cyclodextrins are commonly used for the separation of enantiomers (chiral applications), but have also found use in the separation of geometric and structural isomers (e.g. α-pinene and β-pinene). Cyclodextrins can be used in their native form or as chemically modified additives. By derivatizing the outer rim hydroxyls of the cyclodextrin with various functional groups, the properties of the native cyclodextrins can be changed. Several representative examples of native and derivatized cyclodextrins, together with the types of molecules separated, are outlined in Table 2.

One of the main advantages to derivatization is the change in the solubility properties of cyclodextrins. Native β-CD, which is one of the most commonly used inclusion-type additives, has a very low solubility in aqueous–organic systems (solubility = 1.85 g

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Table 2 Typical applications of native and derivatized cyclodextrins in liquid chromatography

Cyclodextrin	*Abbreviation*	*Applications*
Hydroxypropyl β-CD	HPBCD	Barbiturates, chlorophenols, amino acids, laser dyes, chlorophenols, hydantoins
Carboxymethyl β/γ-CD	CMCD	Steroidal drugs, amino acids
Water-soluble β-CD polymer	SCDP	Pesticides, barbiturates, chlorophenols, nitrostyrenes, tensides, cyclic hydrocarbons
Dimethyl carbamate β-CD	DMP	Radioligands
2,6-di-*O*-methyl β-CD	DIMEB	Barbiturates
Heptakis(2,3,6-tri-*O*-methyl) β-CD	TRIMEB	Tensides
Naphthylethyl carbamate β-CD	NEC-CD	Benzodiazepines, anesthetics, amines, amino acid esters, diuretics
Cationic β-CD		Amino acids, hydantoins
Acetyl β-CD		Anticancer drugs, aminoalkyl phosphonic acids, bronchodilators
Permethylated β-CD		Amino acids
Maltosyl β-CD		Amino acids, alkaloids
Sulfated β-CD		Anesthetics, antiarrhythmics, antimalarials, catecholamines, antidepressants, anticonvulsants, antihistamines
Native α-CD		Barbiturates, ArOH, tensides, terpenes
Native β-CD		Barbiturates, amino acids, PAHs, ArOH, aflatoxins, hydantoins, antiepileptic drugs, tensides, terpenes, alkaloids
Native γ-CD		Barbiturates, tensides, porphyrins, β-blockers

ArOH, aromatic alcohol.

per 100 mL of water, ≈ 16 mmol L^{-1} solution). Solutions of derivatized cyclodextrins, however, can be made at concentrations as high as 400 mmol L^{-1}. It is important to note that these functionalized cyclodextrins are complex mixtures of reaction products. The increased solubility is a direct consequence of this product impurity. Unfortunately, the more concentrated cyclodextrin solutions have increased viscosity. Derivatization of cyclodextrins also affects its chromatographic selectivity, as a result of additional interactions made possible by the presence of various functional groups.

Liquid chromatography Thus far, of all the potential host molecules, cyclodextrins are the most extensively used MPAs in liquid chromatography. Most commonly, cyclodextrins are used as MPAs in the reversed-phase mode. The mobile phase typically consists of an aqueous buffer and an organic modifier. The cyclodextrin additive is included in the buffer component at concentrations appropriate to the application. In general, as the concentration of the cyclodextrin is increased, the enantioselectivity increases until an upper limit is reached. This is illustrated in **Figure 2** for the separation of (±)-α-pinene. The organic component of the mobile phase should not form strong inclusion complexes with cyclodextrin as this will limit the interactions with the analyte(s) of interest. Large, bulky organic solvents should be avoided for this reason. Solvents typically used are methanol and acetonitrile, both of which form relatively weak inclusion complexes with cyclodextrins. Percentages of organic solvent used can be as low as 3% and as high as 45% in an isocratic mixture where gradients are not used. Typically, octadecylsilane (ODS) columns are used but other bonded phases can be employed, depending on the application.

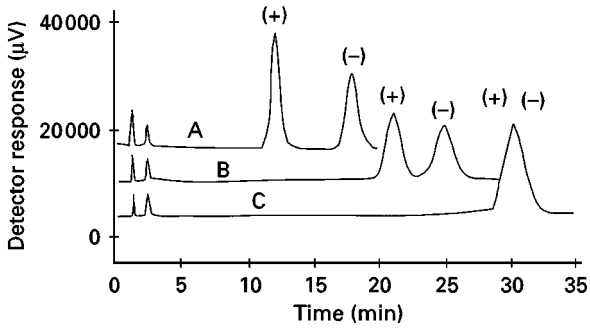

Figure 2 Separation of α-pinene enantiomers using α-CD as a mobile phase additive: (A) 16 mmol α-CD; (B) 8 mmol α-CD; (C) No α-CD. (Adapted from Moeder C, O'Brien T, Thompson R and Bicker G (1996) Determination of stoichiometric coefficients and apparent formation constants for α- and β-CD complexes of terpenes using reversed-phase liquid chromatography. *Journal of Chromatography* A 736: 1–9).

As mentioned, the use of cyclodextrins as MPAs allows the separation of enantiomers (e.g. (±)-α-pinene) and structural isomers. This allows the application of this method to many pharmaceuticals, pesticides and natural products. By varying the experimental parameters (type and concentration of cyclodextrin, percent organic solvent, etc.), information about the strength and stability of the complex can be obtained.

Mechanistic information about complex formation can also be obtained from the data. Frequently, a 1 : 1 host–guest complex is formed, but 2 : 1 complexes have been reported in the separation of terpenes and polycyclic aromatic hydrocarbons (PAHs).

Thin-layer chromatography Cyclodextrins can also be used as complex hosts in TLC. They are almost exclusively employed as MPAs rather than as bonded phases because there are no commercially available cyclodextrin bonded-phase TLC plates. The success of cyclodextrins may be partially attributed to their UV transparency.

Commercially available, reversed-phase plates are typically used. However, a 5% paraffin-in-hexane solution can be used to impregnate standard TLC plates for use as reversed-phase plates. The mobile phase is usually a water/organic solvent mixture with occasional use of buffers in the aqueous portion. As in column chromatography, the organic component is typically methanol or acetonitrile, and in some cases ethanol. The concentration of cyclodextrin in the mobile phase is usually less than 30 mmol L^{-1} and the choice of cyclodextrin concentration is governed by the same factors as discussed above for liquid chromatography. In general, as the concentration of the cyclodextrin is increased, the development time will increase due to the increased viscosity of the resulting solution.

The use of MPAs in TLC is often used to study the binding characteristics of various compounds to inclusion complex host molecules. This is typically accomplished using the following general equation (eqn [1]):

$$R_M = R_{M0} + bC \qquad [1]$$

where R_M is the actual R_M value of an analyte determined at C (mmol L^{-1}) additive concentration; R_{M0} is the R_M value of an analyte extrapolated to zero additive concentration; b is the decrease in the R_M value caused by a 1 mmol L^{-1} increase of the additive concentration in the eluent (indicator of the complexing capacity); and C is the additive concentration in the eluent (mmol L^{-1}).

The slope value, b, is linearly related to the stability of the host–guest complex. For example, a wide variety of pharmaceuticals, nonionic surfactants (tensides) and substituted aromatics have been investigated by this method. Frequently, information is needed about the steric and hydrophobic character of various analytes. Host–guest binding constants can provide information about inclusion within the cyclodextrin cavity. Cyclodextrins have also been used to study the effects of inclusion on the stability and transport of drugs. It is known that cyclodextrins can influence many of the biological properties of drugs when a host–guest complex is formed. The uptake, adsorption properties and degradation of the drug can be modified by complex formation.

Cserháti and Forgács recently studied complex formation between carboxymethyl-γ-cyclodextrin and steroidal drugs. They found that the cyclodextrin mediated the hydrophobicity of the drugs suggesting that the complexed drug may have a modified efficacy.

Macrocyclic Antibiotics

In contrast to cyclodextrins, which have been used for chiral as well as achiral applications, macrocyclic antibiotic MPAs have been applied exclusively to enantiomeric separations. Macrocyclic antibiotics such as vancomycin and erythromycin have been used to resolve enantiomers by TLC. The first macrocyclic antibiotic to be used as a MPA was vancomycin by Armstrong and Zhou in 1994. It was used as a chiral selector in the TLC resolution of dansyl-amino acids, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)-derivatized amino acids, and several pharmaceutical compounds. The composition of the mobile phase as well as the nature of the stationary phase were found to affect chiral resolution for these compounds. The mobile phase consisted of 0.6 mol L^{-1} NaCl with acetonitrile concentration between 17 and 40 vol%. The purpose of NaCl was to stabilize the binder on the TLC plates. For some of the derivatives, a 1% triethylammonium acetate buffer (pH 4.1) was also needed. It was found that diphenyl-type bonded phases provided the best resolution of the compounds studied. For all the amino acid derivatives, the D-enantiomer had a larger R_F value than the L-enantiomer.

Inclusion Complexation in the Stationary Phase

As mentioned previously, no commercially available TLC plates incorporate inclusion complexation ligands. However, conventional TLC plates have been coated with various inclusion host molecules. For example, erythromycin was used to resolve a variety of dansyl-DL-amino acids. By impregnating the plate with 0.05% erythromycin, resolution of the enantiomers of the amino acids was achieved in a relatively short time (20–25 min). A mobile phase consisting of 0.5 mol L^{-1} NaCl, acetonitrile and methanol was used for the separation with one amino acid requiring the presence of acetic acid for separation. The amount of NaCl solution used was generally 7–25 times the amount of acetonitrile used. Methanol was required in very small amounts in some cases. The resolution of the enantiomers was affected by small changes in the mobile phase composition, as is the case with most chiral separations.

Cyclodextrin Phases

Among the inclusion-type stationary phases, the cyclodextrin phases have been the most widely used and the most successful, specifically in the area of chiral separations. Most chiral separations reported on native CD phases (α,β,γ) have been accomplished in the reversed-phase mode using aqueous buffers with small amounts of organic modifier. β-CD is the most commonly used of the native cyclodextrins. The applicability of CD phases has increased with the development of strategies for derivatizing these compounds. Functionalized cyclodextrins have also been used in the reversed-phase, normal-phase, and nonaqueous reversed-phase modes.

The functional groups on derivatized CDs can play a variety of roles in enhancing enantioselectivity. In some cases the substituent provides additional sites for interaction or may enlarge the CD cavity, allowing inclusion of larger analytes. Reported applications of the cyclodextrin phases are seemingly endless. However, several general conclusions can be drawn based on applications along with the theoretical work done in this area. First of all, inclusion complexation is believed to be a significant interaction only when CD-bonded phases, native or derivatized, are used in the reversed-phase mode. Next, inclusion complexation requires that at least some portion of the analyte fits structurally into the CD cavity. Typically, the presence of an aromatic ring in the analyte molecule satisfies this condition. Results indicate that chiral recognition is enhanced if the stereogenic centre lies between two π-systems or is incorporated in a ring. The presence of secondary hydroxyl groups at the rim of the CD cavity allows for hydrogen bonding with polar molecules. Amine and carboxyl functional groups have been shown to interact strongly with those hydroxyl groups on na-

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tive CD phases, while nitrate, sulfate, phosphate and hydroxyl functional groups seem to prefer inclusion. Finally, both flow rate and temperature are known to affect CD-based chiral recognition in the reversed-phase mode. The enantioselective inclusion properties of native CDs have rendered them quite useful as chromatographic substrates, and the ability to derivatize these macrocyclic compounds has made them extremely versatile as inclusion complex host molecules.

Crown Ether Phases

The use of crown ethers as inclusion complexation stationary phases for LC originated with the work of Cram and co-workers during the mid-1970s. They observed that these compounds, specifically tetranaphthylated 18-crown-6-ethers, could form inclusion complexes with alkylammonium compounds and used this idea to develop a chiral stationary phase for the separation of enantiomeric amines. Initially, crown ethers were covalently attached to a silica gel or polystyrene matrix until it was realized that they could be dynamically coated onto reversed-phase packing materials. Eventually, this chiral crown ether stationary phase was made commercially available. Numerous applications have demonstrated the ability of this phase to resolve most primary amino acids, amines, amino esters and amino alcohols. **Figure 3** shows a chromatogram in which a series of three racemic amino acids are separated using an ODS column coated with the chiral crown ether, compound [II]. With respect to enantiomeric separations, the crown ether phase is unique in that it does not require that analytes possess an aromatic ring for the achievement of chiral separation. Optimum performance of the crown ether is observed in acidic mobile phases that ensure protonation of the primary amine functionality, which is included in the crown ether cavity. Typically, perchloric acid ($\cong 0.01\ \text{mol L}^{-1}$) with small amounts of acetonitrile or methanol is recommended as it provides better resolution and exhibits low UV absorption. A notable advantage of the crown ether phases is the commercial availability of both chiral configurations, so that the enantiomeric elution order can be readily changed.

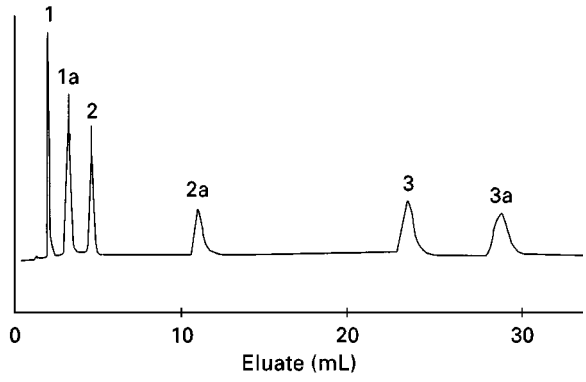

Figure 3 Enantiomeric separation of racemic amino acids: 1, D-arginine; 1a, L-arginine; 2, D-methionine; 2a, L-methionine; 3, D-tyrosine; and 3a, L-tyrosine. (Adapted from Shinbo *et al.*, 1987.)

Calix[*n*]arene Phases

Much like cyclodextrins, calixarenes are macrocyclic compounds that may be easily functionalized, which gives them great potential as stationary phase material. Unlike CDs, however, calixarenes have not yet been extensively employed in liquid chromatography, and therefore only a limited number of applications have been reported. The most successful applications have come with the calixarene chemically immobilized onto a silica gel matrix using a short hydrophillic spacer.

Successful separations of several classes of compounds including amino acids, polyaromatics, nucleosides and nucleobases using a *p*-tertbutyl calixarene-bonded phase indicated that this phase behaves primarily as a reverse-phase material. A typical chromatogram illustrating the separation of nucleosides on this phase is shown in **Figure 4**. In the same study, calixarene phases were reported to form strong inclusion complexes with sodium ions. The complexation with sodium presumably induces a conformational reorientation of the calixarene that increases its hydrophobic surface area, allowing for stronger interaction with hydrophobic analytes.

In another study using a calix[4]arene tetradiethylamide phase, selective retention of Na^+ over other alkali metals and Ca^{2+} over Mg^{2+} was observed using only water as the mobile phase. Furthermore, addition of methanol to the mobile phase resulted in increased retention of NaCl while the retention of LiCl, KCl and CsCl was unaffected by methanol.

Macrocyclic Antibiotic Phases

Stationary phases based on macrocyclic antibiotics (MAs) such as vancomycin and teicoplanin were introduced by Armstrong in 1994. Now commercially available, and used almost exclusively for enantiomeric separations, these phases are typically referred to as the Chirobiotic™ phases. **Figure 5** illustrates a chiral separation of bromocil, devrinol and coumachlor on a vancomycin stationary phase. Chiral selectivity with these phases is demonstrated in

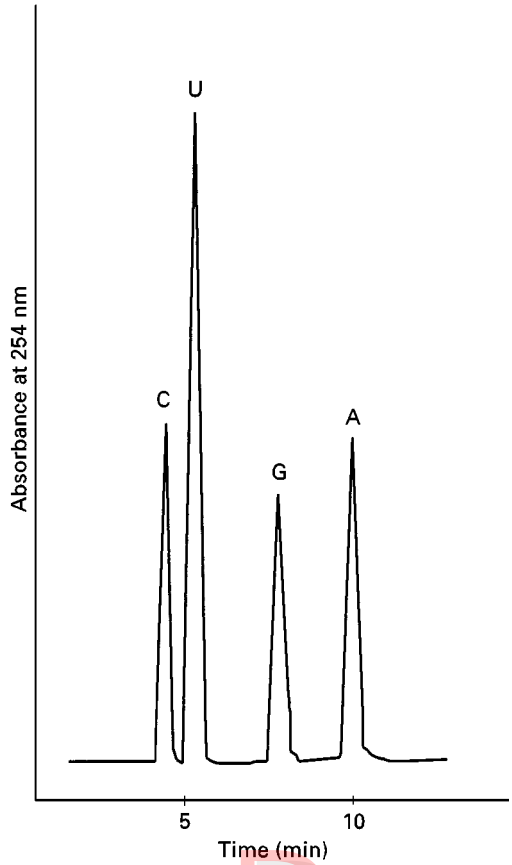

Figure 4 Separation of four nucleosides using a calixarene-bonded phase: cytidine (C), uridine (U), guanosine (G) and adenosine (A). (Adapted from Friebe *et al.*, 1995.)

the normal, polar organic and reversed-phase modes, allowing for the separation of a large variety of chiral analytes. The complexity of the antibiotic structures provides a number of potential interactions that may assist in separation, only one of which is inclusion complexation. Furthermore, an inclusion mechanism is likely to play a role only in the reversed-phase mode, hence this discussion will be limited to the reversed-phase characteristics of these phases.

A great deal of success has been attained with MA phases for the chiral separations of neutral molecules as well as amides and esters. **Table 3** lists the conditions for several representative examples of chiral separations accomplished on a vancomycin-bonded phase.

In terms of optimization and method development, retention can be controlled by adjusting the amount of organic modifier in the mobile phase. Selectivity is controlled by the type of buffer, the type of organic modifier and the pH. Both efficiency and selectivity are affected by the buffer type, ionic strength, flow rate and temperature. Tetrahydrofuran is reported to be the best overall starting organic modifier for efficiency and selectivity, but acetonitrile, methanol and ethanol have also been used with success.

It is important to note that these MAs can also be derivatized to alter enantioselectivity, which will undoubtedly increase the number of potential applications. Furthermore, the relatively small size of these molecules together with the fact that their structures are known increases the feasibility of performing mechanistic studies on chiral recognition.

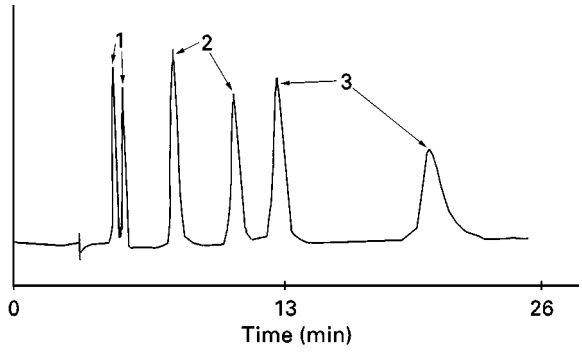

Figure 5 Enantiomeric separation of (1) bromocil, (2) devrinol and (3) coumachlor using a vancomycin stationary phase. (Adapted from Armstrong *et al.*, 1994.)

Conclusions and the Future of Inclusion Complexation in Liquid Chromatography

The inclusion properties of several macrocyclic compounds with guest molecules have been extensively utilized in liquid chromatography. This has been accomplished by employing potential inclusion hosts as mobile-phase additives or as immobilized ligands on the chromatographic stationary phase. The presence of these macrocyclic host molecules in the mobile phase can interfere with analyte detection. Hence, most applications of inclusion complexation in liquid chromatography employ and will continue to employ immobilized ligands. While the commercial availability of the cyclodextrin, macrocyclic antibiotics and crown ether phases has no doubt contributed to their widespread exploitation, the calixarene phases may become more useful in the future as their properties become better understood. Certainly, the calixarene structure is more amenable to intelligent design than the naturally derived macrocyclic antibiotics or cyclodextrins. Inclusion complexation technology has been most extensively used in the area of chiral separations. Derivatization of macrocyclic compounds, such as cyclodextrins, macrocyclic antibiotics, crown ethers and calix[*n*]arenes has further enhanced their chromatographic utility.

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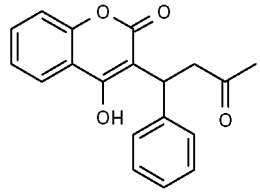
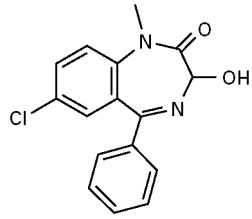
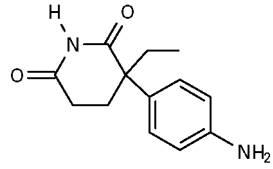
Table 3 Applications of macrocyclic antibiotic-bonded phase

Compound	*Structure*	*k (1st enantiomer)*	*α*	*Mobile phase*	*pH*
Warfarin		1.98 2.27	1.70 1.44	10% ACN/ 90% buffer	7.0 4.1
Temazepam		1.17	1.06	10% ACN/90% buffer	7.0
Aminoglutethimide		0.79	1.15	10% ACN/90% buffer	7.0

The buffer is 1% triethylammonium acetate. The column used was a 25 cm × 4.6 mm i.d. vancomycin stationary phase.

See also: **III/Chiral Separations:** Capillary Electrophoresis; Cyclodextrins and Other Inclusion Complexation Approaches; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Essential Oils:** Gas Chromatography.

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